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<b>(21) International Application Number:</b> PCT/US90/00506 <b>(22) International Filing Date:</b> 29 January 1990 (29.01.90)  <b>(30) Priority data:</b> 316,260                      27 February 1989 (27.02.89)    US  <b>(71) Applicant:</b> BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM [US/US]; 201 West Seventh Street, Austin, TX 78701 (US).  <b>(72) Inventors:</b> NAKAJIMA, Motowa ; 5803 Dryad, Houston, TX 77035 (US). NICOLSON, Garth, L. ; 2611 Valley Manor Dr., Kingwood, TX 77339 (US).		<b>(74) Agent:</b> PARKER, David, L.; Arnold, White & Durkee, P.O. Box 4433, Houston, TX 77210 (US).  <b>(81) Designated States:</b> AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CM (OAPI patent), DE, DE (European patent), DK, DK (European patent), ES, ES (European patent), FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL, NL (European patent), NO, RO, SD, SE, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> METASTASIS-ASSOCIATED COLLAGENOLYTIC METALLOPROTEINASES  <b>(57) Abstract</b>  Disclosed are methods and compositions for testing for various types of human cancers, including in particular metastatic mammary carcinoma, colon carcinoma astrocytoma, renal cell carcinoma, and teratocarcinoma malignant melanoma. The diagnostic method of the invention involves the detection of a gelatinolytic and type IV collagenolytic metalloproteinase having molecular weight of approximately 88 to 92 kilodaltons in size. This high molecular weight type IV collagenolytic enzyme is found to be associated with the cancerous state, but this has not been found in any noncancerous cells tested and further not found in the serum of non tumor bearing animals. The enzyme is associated both with rat and human tumors, the enzyme in each case being quite similar. Methods are disclosed for isolating the 88/92 kd enzyme, the isolated enzyme being useful as a control enzyme and diagnostic assays. Furthermore, the isolated enzyme will be useful in the preparation of antibodies, including monoclonal antibodies, having specificity for the metastasis-associated enzyme. Techniques are also disclosed for isolation and partial purification of the enzyme from both rat and human sources. Furthermore, methods are disclosed for conducting both enzymatic and immunologic based tests for the diagnosis of metastatic and recurrent disease.		

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-1-

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METASTASIS-ASSOCIATED  
COLLAGENOLYTIC METALLOPROTEINASES

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The government may own certain rights in the present invention pursuant to NIH grant numbers RO1-CA41524 and R35-CA44352.

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The present invention relates to metastasis-associated collagenolytic metalloproteinases, and their detection as a means for testing for metastatic or recurrent forms of cancers. In particular, the invention relates to a gelatinolytic and type IV collagenolytic metalloproteinase enzyme having a molecular weight on the order of about 88 to 92 kilodaltons, found to be associated with highly metastatic and other forms of cancer cells.

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Successful malignant cell penetration of basement membranes is an important step in the formation of tumor metastases. Basement membranes are barriers between different tissues that are rigid structures formed from unique macromolecules, such as type IV collagen, laminin, heparan sulfate proteoglycan, and fibronectin. Several proteinases have been implicated in the process of tumor invasion and metastasis, and some are known to be involved in the degradation of basement membrane components (1,2).

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Liotta and coworkers (3) described a type IV collagenolytic proteinase in metastatic tumor cells, and

they found a correlation between type IV collagenase activity and metastatic potential (4). Moreover, previous studies authored by the present inventors have indicated a correlation between the type IV  
5 collagenolytic activities of rat 13762NF mammary adenocarcinoma cells and their spontaneous lung metastatic potentials (5). The metalloproteinase secreted from rat mammary adenocarcinoma cells was found to degrade both alpha-subunits of type IV procollagen and  
10 produce characteristic large molecular weight ( $M_r$ ) fragments (5).

Type IV collagenases have been purified from a variety of mammalian cells, such as metastatic mouse  
15 sarcoma cells (6), human monocytes/macrophages (7), and human H-ras oncogene-transformed bronchial epithelial cells (8). These enzymes are metalloproteinases of  $M_r$  62,000-66,000 in active forms and 66,000-72,000 in latent forms, and some have been found to have gelatinase  
20 activity (8). Latent enzymes have been found to be activated by either trypsin digestion or 4-aminophenyl-mercuric acetate treatment in vitro.

The elevation in expression of certain serum  
25 proteins, such as carcinoembryonic antigen, alpha-feto-protein, and placenta-like alkaline phosphatase, is associated with some human cancers and has been used diagnostically for neoplastic disease (9). In certain  
30 spontaneously metastasizing tumors, an association between increased tissue glycosyltransferase levels and metastasis formation was found, and high levels of glycosyl transferases have been detected in the sera of animals bearing spontaneously metastasizing tumors (9).

35 Prior to now, though, there has been no clear functional relationship, between elevated serum levels of

such components and metastatic disease. Since tissue-degrading enzymes are secreted in high amounts from invasive tumor cells, the levels of degradative enzymes in body fluids should be useful as diagnostic markers of tumor invasion and metastasis. For example, high levels of beta-N-acetylglucosaminidase and beta-glucuronidase were found in the sera of animals and patients with various types of tumors (9). The association of high levels of serum cathepsin B1-like activity with invasive cancer, such as carcinomas of the ovary, vagina, cervix, and breast, has also been reported (10).

The present invention relates to a novel metallo-proteinase, having a  $M_r$  of about 88-92 kilodaltons, that is capable of specifically degrading type IV collagen and gelatin. An important aspect of this metalloproteinase centers on the inventors' finding that its presence is correlated with certain forms of metastatic cancer. The inventors have thus found that the enzyme can form the basis of diagnostic and monitoring assays. The 88/92 kd metalloproteinase has been found to be associated predominantly with highly malignant mammary tumors, breast cancers, colon cancers and malignant melanomas.

The enzyme itself is characterized as a gelatinolytic and type IV collagenolytic metalloproteinase based on its ability to specifically degrade protein substrates including type IV collagen, type I collagen and gelatin. However, this enzyme has not been found to be active in digesting substrates such as fibronectin, albumin, casein, immunoglobulin, or hemoglobin, suggesting that the range of susceptible molecules might be limited. This proteinase is termed a metalloproteinase due to its requirement for a metal ion for activity. For this reason, the enzyme is found to be inactive in the presence of inhibiting concentrations of

chelating agents, such as 10 mM  
ethylenediaminetetraacetic acid (EDTA) or 1, 10-  
phenanthroline. However, the enzyme is not inhibited by  
common proteinase inhibitors such as phenylmethylsulfonyl  
5 fluoride (PMSF) at 2 mM or N-ethyl-maleimide (NEM) at 5  
mM.

The molecular weight range arrived at for this  
enzyme, about 88 to 92 kilodaltons, has been determined  
10 using sodium dodecyl sulfate-polyacrylamide gel electro-  
phoresis (SDS-PAGE) in conjunction with a technique known  
as zymography. Zymography involves identifying the  
location of an enzymatic activity in a gel or similar  
matrix by providing a substrate for the enzyme, -- a  
15 substrate whose interaction with the active enzyme can be  
detected. The enzyme is in this manner directly  
"visualized" in the gel or matrix. Zymography is a  
technique which can be used in the practice of the  
diagnostic method of the invention, in identifying the  
20 enzyme.

The diagnostic method of the invention involves a  
test for human neoplastic disease, particularly  
metastatic disease and tumor recurrence, in a patient  
25 suspected of having such a disease. The method includes  
generally testing for the presence of the  
metalloproteinase in a clinical samples obtained from the  
patient, such as serum, tissue samples, aspirates, and  
other body fluids, but preferably serum. The particular  
30 technique employed to detect the metalloproteinase will  
generally be based on characteristics of the enzyme,  
ranging, for example, from enzymatic characteristics and  
molecular weight, probed by zymography, to its primary  
structure that can be probed immunologically.

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In diagnostic methods based on zymography, a

clinical sample suspected of containing the enzyme is subjected to electrophoresis in a substrate-embedded gel to separate proteins within the sample. The substrate employed for identification of the metalloproteinase will typically be type I or IV collagen, gelatin, or some other suitable substrate the enzyme may be found active upon. In the practice of a zymography-related embodiment, one will generally desire to separate the proteins according to their molecular weights. This allows one to identify the metalloproteinase of the invention according to its molecular weight, at the same time as determining its relative collagenolytic and/or gelatinolytic enzyme activity.

After electrophoresis, the gel is incubated under conditions appropriate to allow any metalloproteinase which may be present in the gel to lyse the substrate. Where one has fractionated the proteins according to molecular weight, it will generally be the case that an SDS-PAGE system of fractionation will have been employed. Under these circumstances, where enzymatic activity is to be detected, one will desire to renature the enzyme, in situ because sodium dodecyl sulfate (SDS) tends to denature proteins and reduce or destroy enzymatic activity. Suitable renaturation can be achieved by rinsing the gel in a dilute nonanionic detergent such as 2.5% Triton X-100, followed by incubation for several hours in an isotonic buffer, such as one containing Tris-HCl and saline in the presence of calcium chloride. Staining of the substrate remaining in the renatured incubated gel, such as through the use of a dye like Coomassie blue, allows one to visualize the enzyme indirectly by means of the appearance of a transparent band against a stained background.

Although one can generally identify the presence of

the metalloproteinase of the invention by reference to the relative migration of molecular weight markers, it will often be desirable to employ a metalloproteinase standard. This allows a direct comparison of a suspected activity with that of a known control, thus improving the overall reliability and sensitivity of the assay. In these situations, one may desire to employ an at least partially purified preparation of the metalloproteinase, for example, isolated by techniques developed by the inventors and disclosed herein.

Alternative to the use of a zymographic approach to diagnoses based on identification of the metalloproteinase enzyme activity/size, the present invention contemplates the use of immunologic approaches. Since immunologic approaches do not require the detection of an enzyme activity, they may ultimately prove more advantageous method than zymography. The immunologic methods contemplated include the use of an antibody, such as a monoclonal antibody, having immunospecificity for the metalloproteinase. Such an antibody can readily be prepared, through the use of a purified preparation of the enzyme, by techniques known in the art, or preferably, techniques found to work well in connection with the development of antibodies against other metastasis-related proteins, such as described in USSN 846,938, incorporated herein by reference.

Suitable immunological techniques would include those which employ a known antibody to detect the presence of an unknown antigen in a clinical sample, whether it be a tissue, cell, aqueous sample or the like, thought to contain the identifying enzyme. It is therefore contemplated that techniques such as ELISA, RIA, Western blot, dot blot, dip stick, ELISA inhibition test, and the like, will prove to be useful techniques



-7-

for identifying the presence of the enzyme in suspected samples.

5       The metalloproteinase of the invention has been  
found to be present in both rat and human sources. While  
it is not clear at present the degree of overall  
similarity of the enzyme from the two species, for  
example, at the amino acid sequence level, there appears  
to be sufficient similarity for them to be considered  
10       functionally equivalent within their respective cells.  
This enzyme likely constitutes a feature of the  
requirement of certain tumor cells, such as metastatic  
cells, to penetrate basement membranes. Its presence in  
such cells, thus, apparently crosses species. It is  
15       proposed that for the purposes of the invention, e.g.,  
use as a control marker for metastasis, enzymes of the  
88/92 kd metalloproteinases class from differing species  
can be used. Of course, for most purposes for use in  
connection with human use or diagnosis, and certainly  
20       where one desires to prepare a preferred antibody, one  
will desire to obtain and employ the human-derived  
material,

25       A metalloproteinase in accordance with an aspect of  
the invention may be obtained from various metastatic rat  
cells and lines, particularly metastatic mammary  
carcinoma cells. The inventors have discovered, for  
example, that highly metastatic clones developed from  
experimental rat mammary adenocarcinoma lines like the  
30       13762NF system (11), provide very good tumors for  
eliciting the serum-borne production of the enzyme in a  
rat host, or even direct production by means of a  
conditioned media approach. Exemplary metastatic rat  
mammary adenocarcinoma lines include the MTLn3 and  
35       MTF7.T35.3 lines, which were derived by clonal selection  
from the 13762NF system (12)

In other embodiments, the 88/92 kd metalloproteinase can be isolated from human sources. The enzyme has been found to be present in a variety of human tumor lines, including human breast adenocarcinoma cells (MCF7; e.g. ATCC HTB22), human colon cancer cell lines (e.g., the KM series, references 13, 14), human malignant melanoma cell lines (e.g., Hs294T, ATCC HTB140, and A375, ATCC CRL1619) human renal cell carcinoma lines (SN series, reference 15) various cell lines derived from human teratocarcinoma PA-1 (16), and even human astrocytoma cell lines (17). Techniques are disclosed herein for obtaining preparations of the enzyme from human sources, generally through the generation of tumor cell-conditioned media, in which cells are grown in a media that is essentially free of extrinsically added serum proteins. The enzyme is provided from the cell-conditioned media in a more purified state by techniques disclosed herein.

Diagnostic kits for testing for the 88/92 kd metalloproteinase of the invention are contemplated generally as taking two forms, depending on whether they are based on enzymatic properties or not, e.g., using zymography or immunology. Zymography-based kits will include one or more reagents employed in carrying out the underlying zymography, e.g., pre-prepared PAGE gels incorporating enzyme substrates, appropriate molecular weight markers, etc., as well as samples of the 88/92 kd control enzyme.

Immunological-based kits, on the other hand, will typically include an antibody having immunoselective binding capability for the 88/92 kd metastasis-associated enzyme, along with means for detecting antibody/antigen reactions. Such immunoreaction detection means are known in the art, and include, e.g., labels or tags such as

enzymes, radioactive ligands, molecular tags, and the like. Often, the detection of a first antibody-antigen reaction may be accompanied by a second antibody, having immunospecificity either for the first antibody, or for second or cumulative epitopic site on the targeted antigen (18). Techniques such as the ABC technique can also be employed. This technique uses unlabeled primary antibody, followed by biotinylated secondary antibody and then a preformed avidin and biotinylated horse radish peroxidase or alkaline phosphatase complex (19).

Figure 1. Zymogram of rat serum type IV collagenolytic metalloproteinases. Blood was withdrawn at various periods of time after injection of 13762NF mammary adenocarcinoma clone cells into the mammary fat pad. Clone MTLn3, MTLn3-T44.5, and MTF7-T35.3 cells were highly metastatic to lung and lymph nodes, whereas MTC cells did not spontaneously metastasize, even after 30 days. Serum (2.5 ul) was dissolved in sample buffer containing sodium dodecyl sulfate and subjected to electrophoresis in a 7.5% polyacrylamide gel embedded with 0.5 mg/ml type IV collagen. After electrophoresis the enzymes were renatured by incubation with 2.5% Triton X-100 in 50 mM Tris-HCl, pH 7.5, and the enzyme reaction was performed at 37°C for 16 h in 0.15M NaCl, 10 mM CaCl<sub>2</sub> and 50 mM Tris-HCl, pH 7.5. Proteinases were detected as transparent bands on the blue background of Coomassie blue-stained slab gels. The strained gels were photographed using Kodak electrophoresis duplicating papers.

Figure 2. Type IV collagenase activity in sera of rats bearing MTLn3 mammary adenocarcinoma. Quantitation of the proteinase activity was estimated by photographing the zymograms using Kodak XAR-5 X-ray films. The active enzyme bands that appeared as positively stained bands on

the X-ray films were scanned for absorbance at 560 nm. The area under each peak that represents proteolytic activity was measured and shown as a relative enzyme activity.

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Figure 3. Relationship between serum  $M_r$  90,000 type IV collagenase activity (in Fisher 344 rats using MTLn3 cells) and number of spontaneous lung metastases. (n=30)

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Figure 4. Zymogram of rat serum gelatinolytic metalloproteinases (see Figure 1 legend).

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Figure 5. Identification of type IV collagenolytic metalloproteinases secreted by cultured human colon carcinoma cells. Colon carcinoma cells, such as KM12C (C), KM12L1 (L1), KM12L4 (L4), and KM12SM (SM), were cultured for 48 h in a serum-free complete medium, and 2-ml aliquots of conditioned media were concentrated and immediately analyzed by electrophoresis in type IV collagen-embedded polyacrylamide gels followed by 24 h incubation and Coomassie blue staining for detection of in situ type IV collagenolytic activity. Enzymes were detected as transparent bands. Ordinate, molecular weight in thousands.

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Figure 6.  $M_r$  90,000 gelatinolytic metalloproteinase in the sera of a series of human breast cancer patients.

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Metastasis, the process by which cancer cells spread from the primary tumor via the circulation to distant organs, is a complex phenomenon requiring many sequential steps. One of the initial steps is the invasion of primary tumor cells into the surrounding host tissues and blood vessels or lymphatic channels (intravasation). Via th circulation, the tumor cells are disseminated to various organs, where they adhere specifically, or ar

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arrested nonspecifically in capillary beds. Subsequent steps include extravasation by invasion through capillary endothelial cells and the subendothelial ECM<sup>3</sup> into the tissues and proliferation of the cells to form secondary tumors. The successful completion of the cascade of events culminating in the formation of metastases depends on the expression of unique properties by progenitor tumor cells, as well as on tumor cell-host interactions (for reviews, see refs. 20-22).

One tumor cell property that is a prerequisite for metastasis is the ability to degrade connective-tissue ECM and basement membrane components, which constitute barriers against invading tumor cells. Indeed, metastatic tumor cells have been shown to produce enzymes such as proteases and glycosidases that are capable of degrading the various components of the ECM (23). One of the proteolytic enzymes, type IV collagenase, which exhibits the ability to degrade type IV collagen, the major structural protein constituent of subendothelial basement membranes, is believed to play an important role in invasion because its levels correlate with the metastatic potential of various transformed and malignant cells (22-23), including rat mammary adenocarcinoma clones derived from the 13762NF cell line (5).

The present invention is directed to a novel, specific metalloproteinase found to be associated with metastatic and recurrent neoplastic disease. The metalloproteinase of the invention is characterized as having a molecular weight of on the order of 88 to 92 kilodaltons as determined by SDS-PAGE/zymography. This particular metalloproteinase exhibits both type I and IV collagenolytic activity, as well as gelatinolytic (denatured type I collagen) activity. The enzyme has not been found to exhibit lytic activity with fibrinectin,

albumin, casein, immunoglobulin, or hemoglobin substrates. These properties suggested that the enzyme is essentially collagen-specific, at least with regard to those substrates which have been tested.

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The 88/92 kd metalloproteinase of the invention may be readily isolated from either rat or human tumor sources which secrete the enzyme, preferably through the preparation of a conditioned media. As those of skill in the art are aware, conditioned medium is medium which has been employed to grow cells which will contain within it biological materials secreted by the cells. Thus, in the case of the metalloproteinase, growth of cells which produce this enzyme in a selected growth medium result in the secretion of the enzyme into the medium, thus providing a ready source for its isolation. Typically, the media employed for the preparation of a conditioned medium will be free of added serum or serum products. This avoids the introduction of an external source of contamination and impurities presented by growth of cells in serum-containing medium.

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For isolation of the metalloproteinase from conditioned media, whether it be from rat or human sources, one will generally desire to first culture the starting cells in serum-containing media for a period of time to allow the cells to begin producing useful amounts of the enzyme. Then, the serum-containing medium is removed and replaced with a serum-free medium. The cells are then allowed to grow on the serum-free medium for a period of time which is selected based on the ability of the cell line or cell source being employed to grow on serum-free medium. This is due to the fact that certain cell lines, and even sublines, tend to grow less well than others under serum-free conditions. This is presumably due to the absence of factors needed for proper growth of cells.

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It will be understood by those of skill in the art that serum-free growth conditions will likely have to be adjusted depending on the cell line or type used for enzyme production.

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After growth of the cells under serum-free conditions for a period of time to allow for secretion of the enzyme into the medium, the medium is harvested and the enzyme present in the medium adsorbed onto a heparin-ligand column, such as a heparin-Sepharose column. It has been found that the 88/92 kd metalloproteinase of the invention binds to such a column when adsorbed at under isotonic conditions, and will elute at a salt concentration range of on the order of about 0.3 to 0.5 M salt. Further purification of the enzyme may then be achieved by subsequent chromatography of the heparin-eluted fraction on gel exclusion matrices such as Sephacry S-200 and/or Sepharose CL-6B.

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In addition to the obtaining of relatively purified preparations of the 88/92 kd metalloproteinase, the invention further concerns a diagnostic assay for cancer based on the presence of this enzyme in clinical samples. Since the enzyme is found to be present in a variety of human tumors, but not in most non-tumor tissues which have been tested, it is proposed that its presence will be diagnostic of a wide variety of cancers. However, in that the enzyme is apparently associated with particular types of cancer, including in particular metastatic and recurrent forms of cancer, the diagnostic identification of the presence of the enzyme will be particularly useful, for example, in the sera of patients suspected of having such forms of cancer.

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One method of identifying the enzyme in samples is through the use of a technique known as zymography,

wherein samples suspected of containing the enzyme are allowed to act upon an enzyme substrate in situ, such as in an electrophoretic gel matrix, which has been impregnated with a substrate of the enzyme. In the case of the present invention, the substrate will typically be gelatin or type IV collagen. The enzyme activity can then be identified by the appearance of bands in the matrix where the enzyme has digested or otherwise acted upon the substrate. A number of general methods are known in the art for conducting zymographic analysis of samples, and such methods can be readily adapted for use in connection with the present invention without undue experimentation in light of the specific disclosure herein.

Another method for diagnostic identification of the enzyme in clinical samples is through the use of immunologic techniques, e.g., through the use of an antibody capable of specifically interacting with and thereby identifying the enzyme. Typically this will be achieved through the preparation of a monoclonal antibody having the desired immunospecificity. It is believed that suitable monoclonal antibodies for use in connection with the invention can be prepared using the techniques herein disclosed.

The examples which follow illustrate various preferred embodiments of carrying out the practice of the invention. It will be appreciated by those of skill in the art that various aspects of these examples are conducted through the use of standard laboratory practices of the inventors in conjunction with techniques developed by the inventors for carrying out the invention. For this reason, it will be apparent that modifications and changes can be made in various of the procedures without departing from the intended spirit and



scope of the invention.

#### EXAMPLE I

5  
Identification of the 88/92 Metalloproteinase  
in a Rat Host, through the use of Rat  
10 Metastatic Mammary Adenocarcinoma Cells

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Highly metastatic clonal lines developed from the  
rat 13762NF mammary adenocarcinoma system were tested for  
their ability to elicit the production of the 88/92  
metalloproteinase. The appearance of the 88/92 kd enzyme  
15 in sera of tumor-burdened animals, or in tumor cell  
conditioned media, was detected through the use of  
zymographic techniques, using either type IV collagen or  
gelatin.

20 The highly metastatic clones which were to be tested  
were first injected subcutaneously into the rat mammary  
fat pad, and allowed to grow. At various time intervals  
following fat pad injection, serum was obtained from the  
rat and tested for presence of the 88/92 kd protein by  
25 zymography. It was generally found to be the case that  
the 88/92 metalloproteinase appeared in rat serum  
approximately 16 or so days following subcutaneous  
injection. Not only was the activity of this high  
molecular type IV collagenase in sera found to correlate  
30 with the extent of metastasis in lung, but the activity  
was not detected in the sera of control rats or rats  
bearing non-metastatic mammary adenocarcinoma cell lines.

35 In addition to being tested for their ability to  
elicit the appearance of the 88/92 kd enzyme in host  
sera, various cells were also tested for their ability to  
produce the enzyme in conditioned media. Thus, it is  
clear that the enzyme is a product of the tumor, and not

the result of some host/tumor interaction.

The particular metastatic cloned lines employed to illustrate this aspect of the invention are designated MTLn3, MTF7-T35.3 and MTLn3-T44.5. The T35.3 and T44.5 lines are highly metastatic subclones of MTF7 and MTLn3 system derivation, described in reference 12. Also tested were relatively non-metastatic lines such as MTLn2 and MTC.

#### CELLS

Rat mammary adenocarcinoma cell clone MTF7-T35.3 was selected by in vitro cloning from MTF7 cells derived from a 13762NF mammary adenocarcinoma tumor growing at a local implant site in the mammary fat pad (12). Clone MTC cells were similarly obtained. The rat mammary adenocarcinoma cell clone MTLn3-T44.5 was cloned in like fashion from MTLn3 cells derived from a 13762NF tumor that spontaneously metastasized from the mammary fat pad to the lung (12).

Cells were grown on 100-mm tissue culture plates (Corning Glass Works, Corning, NY) containing alpha-MEM (GIBCO Laboratories, Grand Island, NY) supplemented with 10% FBS (Biocell, Carson, CA) and no antibiotics at 37°C in a humidified incubator (5% CO<sub>2</sub> and 95% air). In some experiments, MTF7.T35.3 cells were grown in the absence of serum in D-MEM containing 5 ug/ml of insulin, 5 ug/ml of transferrin, 20 uM ethanolamine, 2 mM glutamine, 25 mM selenious acid, 0.2 mM each of 10 nonessential amino acids, and 10 mM N-2-hydroxyethylpiperazine-N'-propane-sulfonic acid buffer, pH 7.5 (serum-free complete medium).

ZYMOGRAMS

Identification of type IV collagenolytic enzymes  
5 secreted by mammary adenocarcinoma cells was performed by  
electrophoresis of serum-free conditioned media in a type  
IV collagen-embedded polyacrylamide gel followed by  
incubation and Coomassie blue staining, based on the  
10 methods described by Heussen and Dowdle (24). Type IV  
procollagen was purified from Engelbreth-Holm-Sworn  
tumors, dissolved in 0.5 M acetic acid or bovine lens  
capsule type IV collagen pepsin fragments (Seikagaku  
America, St. Petersburg, FL) in HCl at pH 3.0,  
15 neutralized with Tris and immediately dissolved in 2%  
SDS. After removing undissolved materials by a brief  
centrifugation at 18,000 x g, SDS-solubilized type IV  
collagen was copolymerized with 7.5% acrylamide. In some  
experiments, each of the following proteins was also  
20 embedded in SDS-polyacrylamide gels: rat hemoglobin; rat  
immunoglobulin G; bovine serum albumin; bovine plasma  
fibrinogen; bovine alpha-casein; and gelatin from swine  
skin (Sigma Chemical, St. Louis, MO). Electrophoresis of  
serum, or other samples such as one obtained from  
conditioned media, was carried out by the method of  
25 Laemmli (25) in polyacrylamide gels containing 0.5 to 1.0  
mg/ml of type IV collagen.

In the case of serum samples, serum (2.5 ul) was  
mixed with the same volume of sodium dodecyl sulfate  
30 sample buffer without reducing agents, and immediately  
subjected to electrophoresis in a 7.5% polyacrylamide gel  
containing the protein substrates. After  
electrophoresis, gels were rinsed twice with 2.5% Triton  
X-100 in 50 mM Tris-HCl buffer, pH 7.5, and incubated at  
35 37°C for 16 h in 0.15 M NaCl, 10 mM CaCl<sub>2</sub>, and 50 mM Tris-  
HCl buffer, pH 7.5, containing 0.05% NaN<sub>3</sub>. Gels were

-18-

stained with 0.05% Coomassie blue, 10% isopropanol, and 10% acetic acid in H<sub>2</sub>O and these destained with 10% isopropanol and 10% acetic acid in H<sub>2</sub>O. Type IV collagenolytic enzymes were detected as transparent bands on the blue background of Coomassie blue-stained slab gels.

The stained gels were photographed using Kodak electrophoresis duplicating papers (Kodak, Rochester, NY). Quantitative analysis of the collagenolytic enzyme activity was achieved by photographing the zymograms using Kodak X-AR-5 X-ray films. The active enzyme bands that appeared as positively stained bands on the X-ray films were scanned for absorbance at 450 nm using a Beckman DU-8 spectrophotometer. The area under each peak that represents collagenolytic activity was measured and shown as a relative enzyme activity.

In order to conduct zymography using samples obtained by the preparation of conditioned media, the following steps were performed. Mammary adenocarcinoma cells ( $1 \times 10^6$  cells) were seeded in a 10-cm tissue culture plate and cultured for 24 h in serum-free complete medium containing 10% heat-inactivated FBS. The cells were extensively washed with DPBS and then further cultured in 10 ml of serum-free complete medium. The serum-free culture supernatants were withdrawn and sequentially centrifuged at  $800 \times g$  and  $18,000 \times g$ , and then aliquots of the supernatants were concentrated with Centricon 30 concentrators (Amicon, Danvers, MA) and mixed with SDS sample buffer.

Cell extracts in Triton X-100 were also prepared to examine the total cellular enzyme activities. Cells ( $1 \times 10^6$ ) were grown as described above, extensively washed with DPBS, and then solubilized in 4°C in 0.2% Triton X-

100 in 50 mM Tris-HCl buffer, pH 7.5. Cell extracts were centrifuged for 5 min at 18,000 x g, and the supernatants were immediately mixed with SDS sample buffer without beta-mercaptoethanol.

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#### Identification of Type IV Collagenolytic Metalloproteinases by Zymograms

10 When the conditioned media from 48 h cultures were analyzed on zymograms, two major proteinases of apparent molecular weights of 88,000 and 64,000 were detected as clear bands. These active enzyme bands did not appear when a chelating agent, such as EDTA or 1,10-phenanthroline, was added to the incubation buffer at a concentra-  
15 tion of 10 mM, whereas phenylmethylsulfonyl fluoride (2 mM) and N-ethylmaleimide (5 mM) did not inhibit the enzyme activity. These results indicate that the  $M_r$  88,000 and 64,000 proteinases are both metalloproteinases. These two proteinase bands were also  
20 observed in gels embedded with gelatin or with pepsin fragments of type IV collagen instead of intact type IV collagen. However, when polyacrylamide gels were embedded with serum albumin, hemoglobin, immunoglobulin G, alpha-casein, or fibronectin instead of type IV  
25 collagen, the  $M_r$  88,000 and the  $M_r$  64,000 bands did not appear. The results indicated that these enzymes are specific for gelatin and might be responsible for the collagenolytic activity.

30 The 88/92 kilodalton type IV collagenolytic metalloproteinase was clearly detectable in the sera of rats bearing metastatic mammary adenocarcinoma cell lines, such as MTLn3 and MTF7.T35.3 (Figures 1 and 4). As can be seen from Figure 1, the presence of the 88/92 kd  
35 enzyme, using a type IV collagen substrate, was readily apparent in the sera of rats by day 16 following fat pad

injection of MTLn3 cells. Similar results were obtained with the other metastatic lines, including MTF7-T35.3 and MTLn3-T44.5. However, no such bands were detectable in the case of the MTC-bearing rats or control rats.

5

A virtually identical result was observed using a gelatin substrate for the zymographic gels (Figure 4). As can be seen in Figure 4, by 16 days post-injection, sera from the MTLn3 bearing rats exhibited a pronounced band corresponding to the 88/92 kd enzyme, using a gelatin substrate. Moreover, similar to type IV collagen-based experiments no corresponding bands of enzyme activity were seen in sera from normal or MTC-bearing rats.

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Type IV collagenase activity levels in sera of rats bearing MTLn3 mammary adenocarcinoma were also measured quantitatively as a function of time (Figure 2). Quantitation of the proteinase activity was estimated by photographing the zymograms using Kodak XAR-5 X-ray films. The active enzyme bands that appeared as positively stained bands on the X-ray films were scanned for absorbance at 560 nm. The area under each peak that represents proteolytic activity was measured and shown as a relative enzyme activity. As can be seen from Figure 2, the enzyme levels began rising after day 10 following fat pad injection, and appeared to peak on about day 30.

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Studies were also conducted to identify the correlation, if any, between increased levels of serum high molecular weight (88/92 kd) type IV collagenase activity with the extent of lung metastases (Figure 3). These experiments were performed as follows: MTLn3 cells were harvested from subconfluent cultures by 0.25% trypsin treatment. Rats received  $1 \times 10^6$  MTLn3 cells/0.5 ml phosphate buffered saline in the 1 ft posterior

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inguinal mammary fat pad. Rats were sacrificed 30 days post-injection of tumor cells using Metofane anesthesia and the blood was withdrawn to prepare serum for the enzyme assay. Rats' bodies were subjected to complete gross necropsies. After fixing lung tissues in neutral buffered formalin, the number of lung surface metastases were measured. The relative activity of 88/92 kd type IV collagenase in serum was determined by zymography as described above. As shown in Figure 3, the results demonstrated a direct correlation between the level of type IV collagenase activity that was measured, and the number of spontaneous tumor lung colonies observed by a dissecting microscope.

#### EXAMPLE II

##### Demonstration of the 88/92 kd Type IV Metalloproteinase in Human Tumors

A number of human tumors, including colon carcinoma cell lines, malignant melanoma cell lines, renal cell carcinoma lines, human breast cancer lines, teratocarcinoma cell lines and the sera of malignant breast cancer patients, were tested for the presence of the 88/92 kd metalloproteinase. The cell lines were tested zymographically for the 88/92 kd enzyme through the use of conditioned media, whereas the cancer patients were tested through the use of their sera.

The colon carcinoma lines tested were derived from both primary human colon carcinomas (HCC) (KM12) and KM20 series), as well as from a liver metastasis (KM23 series). The development of these lines is described by Morikawa et al. Highly metastatic subclones were obtained by passage through nude mice and selection. The

techniques employed for HCC testing were as follows:

#### Mice

5           Male athymic BALB/c nude mice were obtained from the Animal Production Area, NCI-Frederick Cancer Research Facility (Frederick, MD). Mice were used when 8 weeks old and were maintained in a laminar flow cabinet under specific pathogen-free conditions.

10

#### Tumor Lines

          Fresh tumor specimens from primary HCC (KM12:Dukes' B<sub>2</sub> and KM20: Dukes 'D) and from a liver metastases (KM23: Dukes'D) were obtained at surgery from 3 different patients. Tumor tissue was enzymatically dissociated with collagenase (type I, 200 units/ml) and DNase (270 units/ml) (Sigma Chemical Co., St. Louis, MO). The cell suspension was filtered through a 4-layer sterile gauze and washed 3 times in serum-free medium. The procedure yielded a suspension of mainly single tumor cells or small clumps of cells (<5) with a viability of <80% (trypan blue exclusion). Suspensions of  $2 \times 10^6$  viable cells were established in culture and injected into the subcutis or spleen of different nude mice. Eight to twelve weeks later, the mice were killed. Local growth (tumorigenicity) and distant growth (liver nodules) were determined.

#### 30           Selection of Highly Metastatic Cells from the KM12C Line

          Subsequent to establishment in culture, cells of the KM12C (Dukes' B) were implanted into the spleen of nude mice. Solidary lesions from the liver (experimental metastases) were isolated and established in vitro

35



(KM12L1 line). This cycle was repeated four times to yield line KM12L4 as described (14). Cells of parental KM12C were also injected into the cecum of nude mice. When the mice became moribund, they were killed, a spontaneous liver metastasis was harvested, established in culture, and designated as KM12SM line. All the lines were shown to be of human origin by karyotype analysis and isoenzyme determinations (Authentikit, Corning Medical, Corning, NY).

#### Enzymography

Identification of type IV collagenolytic enzymes secreted by colon carcinoma cells was performed by electrophoresis of serum-free conditioned medium in a type IV collagen-embedded polyacrylamide gel followed by incubation and Coomassie blue staining, according to methods described by Heussen and Dowdle. Bovine corneal type IV collagen (Seikagaku America, St. Petersburg, FL) in HCl was neutralized with Tris, immediately dissolved in 2% sodium dodecyl sulfate, and then copolymerized with 7.5% acrylamide. Colon carcinoma cells ( $5 \times 10^6$ /10 ml medium) were seeded in a 10-cm tissue culture plate and incubated in medium containing 10% fetal bovine serum for 24 h. The cells were extensively washed with phosphate-buffered saline and then further grown in 10 ml of serum-free medium for 48 h.

The serum-free culture supernatants were withdrawn and sequentially centrifuged at 800 x g and 18,000 x g. Next, 2-ml aliquots were concentrated by using Centricon 30 concentrators (Amicon) and mixed with sodium dodecyl sulfate sample buffer. Electrophoresis was carried out by the method of Laemmli in 0.5 mg/ml type IV collagen-embedded polyacrylamide gels. After electrophoresis, gels were rinsed twice in 2.5% Triton X-100, 50 mM Tris-

-24-

HCl, pH 7.5, and incubated at 37°C for 16 h in 0.15 M NaCl, 10 mM CaCl<sub>2</sub>, 50 mM Tris-HCl, pH 7.5, containing 0.05% NaN<sub>3</sub>. Gels were stained with 0.005% Coomassie blue and destained in 10% isopropanol and 10% acetic acid, and type IV collagenolytic enzymes were detected as transparent bands on slab gels.

### Results

#### 10 Human Colon Carcinoma

The four HCC cell lines were assayed for type IV collagenolytic activity. During the first 24 h incubation, KM12C, KM12L1, KM12L4 and KM12SM degraded 0.38, 0.62, 0.75, and 0.70 ug of type IV collagen, respectively. A similar degradation tendency for type IV collagen was observed at 48 h. During this period, KM12L4 and KM12SM degraded more type IV collagen than did KM12C and KM12L1 (1.25 and 1.20 versus 0.69 and 0.85 ug/10<sup>4</sup> cells, respectively). The background release was 0.11 ug of type IV collagen degraded by serum proteases. The relationship between type IV collagenolytic activity and metastatic potential of the colon carcinoma cell lines was examined by comparing the type IV collagenolytic activity with the median number of hepatic metastases produced by cells injected into the spleen. After subtracting the background release, the amounts of type IV collagen degraded by colon carcinoma cells during the 24 h and 48 h incubations were plotted against the number of liver tumor colonies. The correlation coefficient (r) and probability (P) are as follows: 24 h, r = 0.809, P = 0.1914; 40 h, r = 0.985, P = 0.0148.

The metastatic potential and type IV collagenolytic activity were also analyzed by the paired group t test. A significant difference between the low metastatic KM12C

cells and the high metastatic KM12L4 and KM12SM cells (P<0.05) was found. The serum-free conditioned media were analyzed by enzymography to identify the proteinases responsible for type IV collagenolysis. The active  
5 proteinases of  $M_r$  98,000, 92,000, 80,000, 68,000, and 64,000 were shown as transparent bands on a type IV collagen-embedded polyacrylamide gel after Coomassie blue staining, with the 92 and 68 kd species being the most  
10 widely apparent in all of the lines (Figure 5). Relative enzyme activity, measured by densitometry, were 1.00, 3.90, 4.33, and 3.89 for KM12C, KM12L1, KM12L4, and KM12SM, respectively. A proteinase of  $M_r$  68,000 was only  
15 detected in the media conditioned by highly metastatic cell lines such as KM12L4 and KM12SM. The type IV collagenolytic activities of the HCC cell lines therefore correlated with their potential to produce experimental  
hepatic metastases after intrasplenic implantation.

20 Human Breast Cancer Malignant Melanoma Renal Cell Carcinoma, Teratocarcinoma, and Astrocytoma Lines

Similar results were obtained in studies employing human malignant melanoma cell (MMC), human breast cancer  
25 renal cell carcinoma, teratocarcinoma, and astrocytoma lines. Well known MMC lines, such as A375 (ATCC CRL1619), A375M, and Hs294T (ATCC HTB140), a breast cancer line MCF7 (ATCC HTB22) and astrocytoma lines, such  
as D54MG, U373MG, and U251MG, were obtained from various sources and tested as described above. Human renal  
30 carcinoma lines SN series were established by in vitro cultures of the primary tumor tissues (SN12C) or its transplate in the kidney of a nude mouse (SN12K), and selected for metastasis from the kidney to the liver (SN12L1) or the lung (SN12M6, SN12M7) in nude mice (15).  
35 Human PA-1 cells at four stages of tumor progression established by Tainsky et al. (16) were also tested for

88/92 kd metalloproteinase activity. These PA-1 cells includes 1) nontumorigenic, 2) preneoplastic and susceptible to transformation by single oncogenes, 3) tumorigenic, and 4) metastatic cells. In particular, these lines were employed to prepare conditioned media as described above for the HCC lines. The results demonstrated the appearance of the 88/92 kd enzyme in the media conditioned by all lines tested except for U373MG and U251MG cells. Furthermore, the enzyme levels were found to correlate with the tumorigenicity, invasiveness, or metastatic potential of the respective line. Among those cell lines the highly metastatic renal cell carcinoma cells (SN12L1, SN12M7) expressed the highest enzyme activities.

#### Human Malignant Breast Cancer Patients

Sera obtained from a series of malignant breast cancer patients were tested for the presence of the 88/92 kd metalloproteinase by zymography. In all more than 30 breast cancer patients were tested. The samples used in this preliminary study were collected 4 to 6 years ago and stored at -20°C. Various of the results are shown in Figure 6, which demonstrate that of 12 of the patient's sera tested in the study shown, 9 tested positive for the 88/92 kd enzyme. The samples which did not exhibit the enzyme were probably inactivated during the storage.

#### EXAMPLE III

##### Partial Purification of the 88/92 kd Metalloproteinase from Rat and Human Sources

Studies have also been conducted wherein it has been determined that the 88/92 kd metalloproteinase can be

readily obtained in useful amounts from conditioned media of either human or rat cells, such as the cell lines discussed above. In particular, the high molecular weight type IV collagenase has been isolated and  
5 partially purified through the use of both MTLn3 tumor-bearing rat serum, as well as from serum-free media conditioned by human breast cancer MCF7 cells. In general, where one desires to obtain larger quantities of the collagenase, one will desire to employ the  
10 conditioned media approach, which is also applicable to isolation of the enzyme from rat sources. The partial purification procedure employed heparin-Sepharose chromatography, in conjunction with gel filtration on column material such as Sephacryl S-200 or Sepharose CL-  
15 6B columns. Using such an approach, the inventors have found that an overall purification of on the order of 1 to 200 can routinely be achieved. Subsequent analyses of the partially purified material by SDS-PAGE techniques reveal that the material is more than 30% pure.

20 For isolation from rat serum, the following technique was employed. A rat received  $1 \times 10^6$  MTLn3 cells injected into the mammary fat pad. Approximately 30 days following subcutaneously injection of cells, the  
25 rat was sacrificed and the serum from the rat collected. Approximately 2 ml of sera was passed on a heparin-Sepharose column (bed volume 10 ml), the serum enzyme being retained on the column in a 0.15 M sodium chloride, 10 mM phosphate buffer at pH 7.2. The column was eluted  
30 with a gradient of 0.15-1.0 M sodium chloride and 10 mM phosphate buffer, pH 7.2. The active fractions eluted at a concentration range of 0.3-0.5 M sodium chloride. These fractions were collected and dialyzed against 0.1% sodium dodecyl sulfate in Dulbecco's phosphate-buffered  
35 saline, pH 7.2, for 3 h length of time and two changes of buffer. The dialyzed material was then concentrated and

about 1 ml loaded onto a Sephacryl S-200 column (1 x 120cm) equilibrated and eluted with the same buffer. The enzyme activity remained in the void volume of this column.

5

The enzyme fraction collected off of the S-200 column was further purified by Sepharose CL-6B gel chromatography, using Tris buffer. The enzyme was eluted in fractions of  $K_{av}$  0.4-0.5 in 50 mM Tris-HCl, 0.15 M sodium chloride, pH 7.5, containing 0.1% sodium dodecyl sulfate. The fractions exhibiting activity on zymograms were pooled and the buffer was replaced with 50 mM Tris-HCl, 0.2 M sodium chloride, 10 mM calcium chloride, 1 mM zinc chloride, pH 7.5, containing 0.5% Brij 35, and the fractions were stored at 4°C.

15

Partially purified fractions were tested for activity using a collagenolytic assay as described by Nakajima et al. (5). In general, suspensions of [<sup>3</sup>H]-acetylated collagens were incubated at 27°C with the partially purified enzyme in 50 mM Tris-HCl, 0.1 M sodium chloride, 10 mM calcium chloride, pH 7.5, containing 0.05% Brij35, type IV collagen was cleaved into smaller fragments, but type I collagen was not degraded. Thus, the high molecular type IV collagenase has a preference for type IV collagen and probably gelatin. The results of these experiments demonstrated that the enzyme had been isolated and partially purified.

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The high molecular weight type IV collagenase was also purified from rat sources using the serum-free medium conditioned by MTF7.T35.3 cells using the same procedures, with the serum-free media being prepared as described above in Example I.

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From the foregoing studies it became apparent to the

inventors that metalloproteinases having molecular weight of on the order of 88-92 kd capable of degrading type IV collagen and gelatin should prove to be useful markers for assessing the potential for tumor metastasis and for detecting a recurrence and monitoring patients that have had their primary tumors removed.

#### EXAMPLE IV

##### Preparation of Monoclonal Antibodies Against the 88/92 kd Metalloproteinase

Monoclonal antibodies are produced by the following methods: The partially purified enzyme is subjected to polyacrylamide cell electrophoresis in the presence of sodium dodecyl sulfate and the enzyme band is isolated therefrom, e.g., by excision of a gel slice, and electro-eluted. The purified enzyme is made into an emulsion with Freund's complete adjuvant and subcutaneously injected into BALB/c mice (200 ug antigen/mouse). The mice are boosted every two weeks i.p. with immunogen and Freund's complete adjuvant. After the third immunization the injection is administered intravenously without adjuvant. The mice are sacrificed 3-4 days after the last immunization, and their spleen is removed aseptically.

The spleen is gently teased over a screen to obtain a single cell suspension, followed by harvesting and washing of the lymphocytes. The splenocytes and P3X myeloma cells (ATCC CRL 1580) are mixed at a ratio of 1:1, pelleted by centrifugation, and polyethylene glycol added over a period of one minute with constant agitation at 37°C. After further stirring, medium is added and cells then washed. The resuspended fusion mixture is

aliquoted into 96 well microtiter plates. Feeder cells prepared from new born mouse thymus are used at the initial low densities of the hybrids. The cells are then repeatedly fed HAT medium beginning the next day, and after 1-2 weeks, if growth is substantial, the supernatant media is screened for antibody production using ELISA.

The antibody specificity is checked by the immunoblotting analysis described below. The resulting positive hybridomas are grown in defined serum-free medium which will allow easier purification of the monoclonal antibodies. The monoclonal antibodies are purified by ammonium sulfate precipitation and chromatography over protein A-agarose (Affi-Gel Protein A MAPS II Kit, Bio-Rad) and Sephadex G-25 columns.

#### EXAMPLE V

##### Immunologic Test For the 88/92 kd Metalloproteinase

##### ELISA Inhibition Test

In the enzyme assays of sera and other body fluids, ELISA techniques will preferably be employed. For preparation of the serum sample, blood is withdrawn by venipuncture without anticoagulant and allowed to clot for about 1 h at 22°C. The clotted sample is centrifuged at 4°C for 10 min at 800 x g and for 15 min at 1,600 x g. The resultant sera is divided into small aliquots and snap-frozen in liquid nitrogen, and then maintained at -80°C until analyzed. A micro ELISA inhibition test will preferably be employed for serodiagnosis of cancer (see, e.g., ref. 26).



A proposed immunologic screening method is as follows. Microtest trays are coated with about 10 ug/ml 88/92 kd metalloproteinase in 0.1 M sodium bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. The trays are blocked by the addition of 1% BSA in bicarbonate buffer for 1 h at 37°C. The wells are aspirated, and the trays are frozen at -76°C until ready for use. A 3 ul aliquot of serum from a cancer patient or a normal donor is added to each well, and then antibody solution (3 ul) diluted appropriately in 0.05% Tween-20 in 1% BSA in PBS, pH 7.4 is added. The trays are incubated for 2 h at 37°C, washed twice with 0.05% Tween-20 in PBS, and aspirated. Anti-mouse immunoglobulin conjugated to horse radish peroxidase solution (0.1 ug) in PBS containing 1% BSA is added to each well, and then the trays incubated for 1 h at 37°C. After washing twice with 0.05% Tween-20 in PBS, 5 ul of O-phenylenediamine substrate is added and incubated for 15 min. The reaction is terminated by the addition of 5 ul of 2.5 M sulfuric acid.

The optical density is measured with a microplate reader, such as a Titertek Multiskan (Flow Labs.). Percent inhibition is calculated for each OD value with use of the following formula:  $\% = (1 - OD/P_{avg}) \times 100$ , where % = percent of inhibition,  $P_{avg}$  = average OD reading of the control sera. The inhibition of binding of antibodies on a linear scale is plotted against the concentration of purified 88/92 kd metalloproteinase added on a log scale. Using this standard curve, the concentration of the 88/92 kd metalloproteinase in serum can be determined. The relationship between serum 88/92 kd metalloproteinase levels and stages of disease can be studied by examining the patient records.

Immunoblotting

An immunoblotting technique is employed to detect enzymes present in cells and tissues. It is believed that this technique will allow one to determine the amount of 88/92 kd metalloproteinase in active and inactive forms. Proenzymes and partial cleavage products may be detected. Immunoreactive proteins are visualized as bands at certain molecular weights and the quantitative analysis of positive protein bands is performed by densitometric scanning.

Equal aliquots (100 ug protein) of cell extracts or tissue extracts are subjected to SDS-polyacrylamide gel electrophoresis on a 5-15% gradient gel or 7.5% gel. The proteins are then electroblotted from the gel to nitrocellulose filter paper (0.1 um pore size) for 2 h at 250 mA in chilled transfer buffer containing 20 mM Tris, 10 mM glycine, pH 9.15, using an electroblotting apparatus. The blots on the nitrocellulose paper are fixed in 50% aqueous isopropanol for 30 min, then rinsed in TST buffer (0.1% Tween 20, 200 mM NaCl, in 20 mM Tris-HCl, pH 7.5). The paper is then incubated for 3 h at 37°C in TST buffer containing 3% BSA, followed by a 3 h incubation with biotinylated antibodies at an appropriate concentration.

After incubation, the paper is rinsed three times in the same buffer and incubated in 5 mM HEPES, 200 mM NaCl, 0.1% Tween 20, pH 7.4 (HST) containing Streptoavidin-alkaline phosphatase for 1 h at 25°C. The paper will be washed three times with TST and three times with water, and developed with 0.16 mg/ml 5-bromo-4-chloro-3-indoyl phosphate and 0.33 mg/ml Nitroblue tetrazolium in 0.1M NaCl, 50 mM MgCl<sub>2</sub>, 0.1 M Tris-HCl, pH 9.5. The paper will be washed several times with water to stop the reaction, and air-dried. The positive bands which appear

on the nitrocellulose paper are analyzed by densitometric scanning, e.g., with video-enhanced computer imaging and analysis.

5           It may also prove appropriate to use dot blot techniques for simple quantitative analysis of 88/92 kd metalloproteinase present in tissue and serum samples. In this technique, tissues are homogenized and solubilized with 0.1% SDS, 0.1% Triton X-100, 0.1% sodium  
10 chorate, 5 mM NEM, 1 mM PMSF in 0.15 M NaCl, 50 mM Tris-HCl buffer, pH 7.5, and centrifuged at 30,000 x g for 30 min. The supernatants are blotted on a nitrocellulose paper using a Bio-Dot microfiltration apparatus (Bio Rad). The serum samples are diluted with 0.1% SDS in  
15 phosphate buffered saline 10-fold and blotted on a nitrocellulose paper. Quantitative detection of antigen is performed as described above.

#### Immunoperoxidase and Immunofluorescence Labeling

20           Studies on enzyme production and localization in various tissues and cells will be performed by immunostaining techniques. To investigate differences between primary tumors and their metastases, and the  
25 heterogeneity of heparanase production among malignant cells in tumor tissues immunoperoxidase and immunofluorescence labeling will be used. The results will be also compared with the stages of disease referred to in the patient records.

30           In immunostaining, paraffin sections and frozen sections of tumor tissues can be stained by immunoperoxidase techniques using purified antibodies and the Vectastain ABC kit (Vector Laboratories, Burlingame, CA)  
35 or goat anti-rabbit or mouse IgG conjugated with horse radish peroxidase. Immunoperoxidase staining is

conducted according to the manufacturer's instructions, e.g., as provided by Vector Laboratories, and the stained specimens are examined on a light microscope.

5 Immunoperoxidase staining can also be employed to examine intracellular and cell surface localization of 88/92 kd metalloproteinase. To see intracellular localization of 88/92 kd metalloproteinase, cells are permeabilized by acetone treatment prior to staining.

10 An alternate method, immunofluorescence labeling, is performed by the indirect immunostaining technique described using affinity-purified 88/92 kd metalloproteinase antibody (primary antibody) and F(ab')<sub>2</sub> fluorescein-conjugated antibody fragments specific for  
15 the primary antibody (secondary antibody). The immunofluorescence-labeled specimens is examined on a UV-equipped light microscope (fluorescence microscope).

20

\* \* \*

It will be appreciated by those of skill in the art that the foregoing description of the invention has been  
25 made in terms of preferred embodiments, including laboratory techniques found by the inventors to work well in connection with the practice of various aspects of the invention. It will be therefore apparent to those of skill in the art, in light of the present disclosure,  
30 that various modifications and changes may be made in the practice of the invention without departing from the spirit and scope of the appended claims. All such modifications and changes are intended to be included in the scope of the invention.

35

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CLAIMS:

1. A gelatinolytic and type IV collagenolytic metallo-  
proteinase exhibiting a molecular weight of between about  
5 88 and 92 kilodaltons upon being subjected to sodium  
dodecyl sulfate-polyacrylamide gel electrophoresis.
2. The metalloproteinase of claim 1, obtained by growth  
10 of metastatic mammary carcinoma cells characterized by an  
ability to secrete the metalloproteinase.
3. The metalloproteinase of claim 1, obtained by growth  
15 of colon carcinoma, renal cell carcinoma, malignant  
melanoma, teratocarcinoma, or astrocytoma cells, each  
characterized by an ability to secrete the metallo-  
proteinase.
- 20 4. The metalloproteinase of claim 1 or 2, further  
identified as a rat metalloproteinase.
- 25 5. The metalloproteinase of claim 1 or 2, further  
identified as a human metalloproteinase.
- 30 6. The method for providing a metalloproteinase in  
accordance with claim 1, the method comprising culturing  
a human tumor cell which secretes the metalloproteinase  
to form a conditioned medium, and obtaining the metallo-  
proteinase from the conditioned medium.
- 35 7. The method of claim 6, wherein the human tumor cells

-41-

comprises metastatic mammary carcinoma cells, colon carcinoma cells or a malignant melanoma cells, renal cell carcinoma cells, astrocytoma cells, or teratocarcinoma cells.

5

8. The method of claim 6, wherein the human tumor cell comprises HS294T malignant melanoma cells.

10

9. A method for providing a metalloproteinase in accordance with claim 1, the method comprising preparing the metalloproteinase from the serum of a rat bearing a metastatic mammary adenocarcinoma.

15

10. The method of claim 9, wherein the metastatic mammary adenocarcinoma comprises a metastatic clone of 13762NF mammary adenocarcinoma cells.

20

11. An antibody against the metalloproteinase of any one of claims 1 through 5.

25

12. The antibody of claim 11, further identified as a monoclonal antibody.

30

13. A method for testing for human metastatic disease or tumor recurrence in a patient suspected of having such a disease, the method comprising testing for the presence of a metalloproteinase as defined by claim 1 in a clinical sample obtained from the patient.

35

14. The method of claim 13, wherein testing for the presence of the metalloproteinase includes the steps of:

5           subjecting a clinical sample to electrophoresis  
          in a substrate-embedded gel to separate  
          proteins within the sample;

          incubating the gel under conditions appropriate to  
          allow any metalloproteinase as defined by claim  
10       1 which may be present in the gel to lyse the  
          substrate; and

          identifying the presence of such a metallo  
          proteinase in the gel following such an  
15       incubation, the presence of the metallo-  
          proteinase being indicative of the presence of  
          the disease.

20       15. The method of claim 14, wherein the substrate  
          comprises type I collagen, type IV collagen or gelatin.

25       16. The method of claim 14, wherein the gel comprises a  
          polyacrylamide gel.

30       17. The method of claim 14, wherein identifying the  
          presence of such a metalloproteinase includes comparing  
          any metalloproteinase identified with a standard metallo-  
          proteinase as defined by claim 1.

35       18. The method of claim 13, wherein testing for the  
          presence of the metalloproteinase comprises testing the  
          sample immunologically through the use of an antibody as

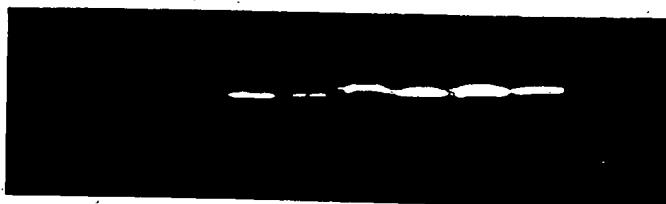
defined by claim 11.

- 5 19. A kit for testing for the presence of a metallo-proteinase in a biological sample, the kit comprising anti-metalloproteinase antibody in accordance with claim 11, and means for detecting an immunoreaction between such antibodies and the metalloproteinase.

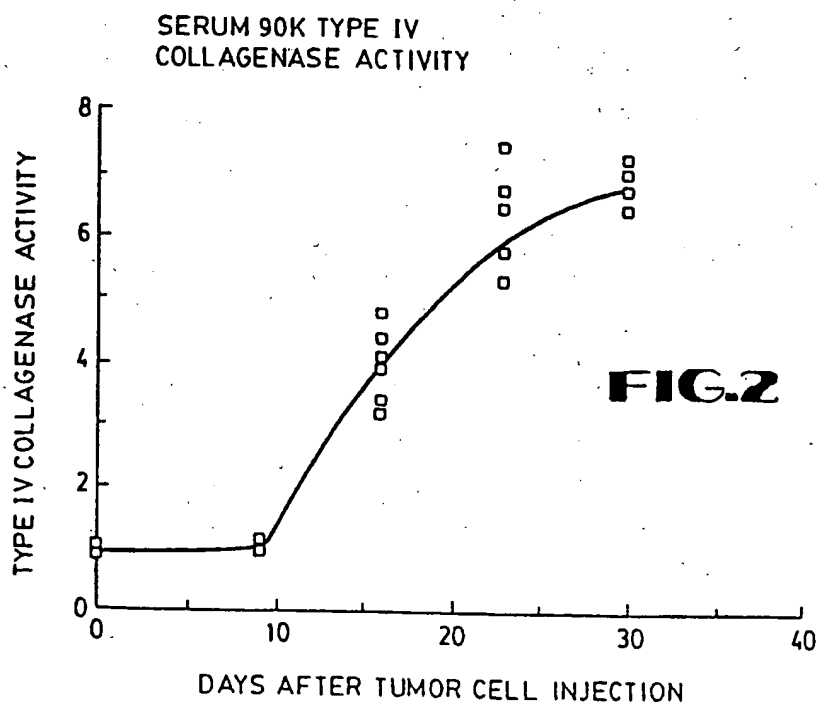
1/3

**FIG.1**

DAYS / CLONE  
 30/NORMAL  
 30/NORMAL  
 30/MTC  
 30/MTC  
 30/MTF7-T35.3  
 30/MTF7-T35.3  
 30/MTLn3-T44.5  
 30/MTLn3  
 23/MTLn3  
 16/MTLn3  
 9/MTLn3  
 0/MTLn3



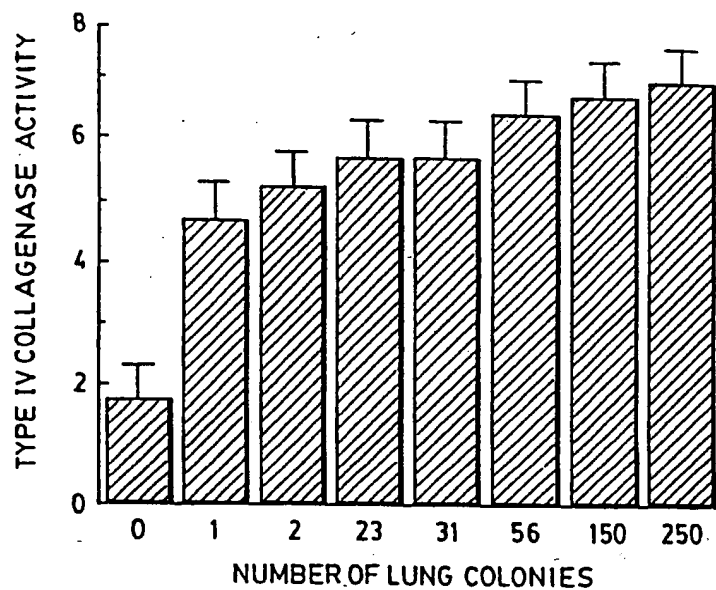
TYPE IV COLLAGEN



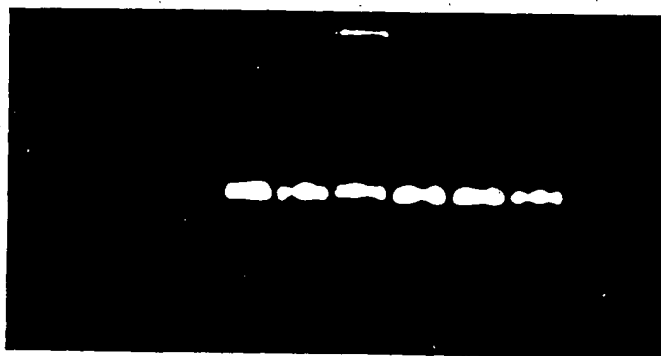
**FIG.2**

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2/3

**FIG.3**

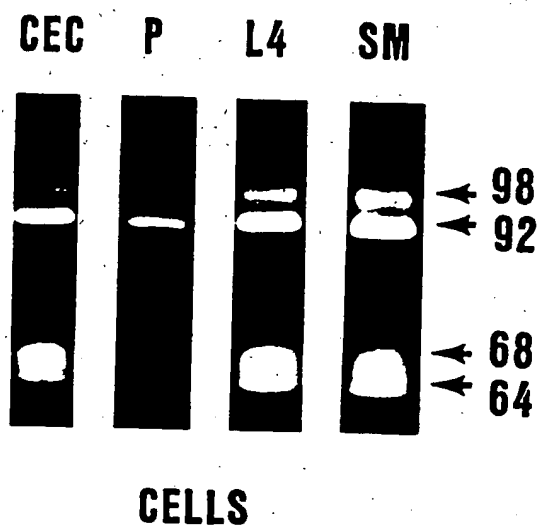
DAYS / CLONE  
30 / NORMAL  
30 / NORMAL  
30 / MTC  
30 / MTC  
30 / MTF7-T35.3  
30 / MTF7-T35.3  
30 / MTLn3-T44.5  
30 / MTLn3  
23 / MTLn3  
16 / MTLn3  
9 / MTLn3  
0 / MTLn3

**FIG.4**

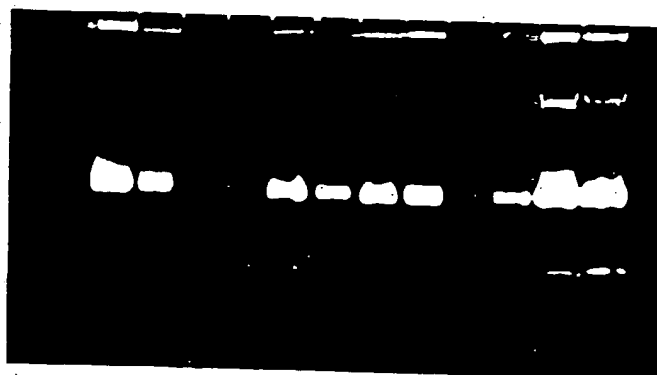
GELATIN

SUBSTITUTE SHEET

**FIG.5**



A B C D E F G H I J K L



**FIG.6**

SUBSTITUTE SHEET



# INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/00506

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup> According to International Patent Classification (IPC) or to both National Classification and IPC <b>IPC5: C 12 N 9/64, C 12 Q 1/37</b>		
<b>II. FIELDS SEARCHED</b> Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
IPC5	C 12 N	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>		
Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, Vol. 154, No. 3, 1988, M. Ballin et al: "Ras Oncogene Mediated Induction of a 92kDa Metalloproteinase; Strong Correlation with the Malignant Phenotype ", see page 832 - page 838	1-12
Y	--	13-19
Y	THE CANCER BULLETIN, Vol. 39, No. 3, 1987, Motowo Nakajima et al: "Basement Membrane Degradative Enzymes and Tumor Metastasis ", see page 142 - page 149 see page 146 right column	13-19
<p><sup>10</sup> Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&amp;" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search <b>15th May 1990</b>		Date of Mailing of this International Search Report <b>- 5. 05. 90</b>
International Searching Authority <b>EUROPEAN PATENT OFFICE</b>		Signature of Authorized Officer <b>F.W. HECK</b>

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	NATURE, Vol. 284, 1980, L.A. Liotta et al: "Metastatic potential correlates with enzymatic degradation of basement membrane collagen ", see page 67 - page 68  --	1-19
A	Chemical Abstracts, volume 109, no. 11, 12 September 1988, (Columbus, Ohio, US), Tsuda, Michio et al : "High molecular mass type IV collagen-specific metalloprotease from human carcinoma tissue ", see page 295, abstract 88532c, & FEBS Lett. 1988, 232( 1), 140- 144  --	1-19
A	Chemical Abstracts, volume 111, no. 23, 4 December 1989, (Columbus, Ohio, US), see page 448, abstract 212922n, & US., 196242 (Liotta, L.A. et al) 1988  --	1-19
A	Chemical Abstracts, volume 111, no. 23, 4 December 1989, (Columbus, Ohio, US), Nethery, Andrew et al : "Identification, partial purification and characterization of high-molecular-weight gelatin-degrading metalloproteinases produced by a rat mammary carcinoma cell line ", see page 419, abstract 212652z, & Biochim.Biophys.Acta 1989, 993( 1), 42- 47  --	1-19
A	Chemical Abstracts, volume 110, no. 15, 10 April 1989, (Columbus, Ohio, US), Nethery, Andrew et al : "Identification of a metalloproteinase copurifying with rat tumor collagenase and the characteristics of fragments of both enzymes ", see page 317, abstract 131003r, & Biochim.Biophys.Acta 1989, 994( 2), 149- 160  -- -----	1-19